In vitro studies on radiation and temozolomide in human glioma

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Chapter 1

General introduction

Contents

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1.1 Radiation biology

In 1895, Wilhelm Conrad Röntgen in Germany discovered a new type of electrically generated radiation, X-rays that could penetrate through the body and caused blackening of photographic paper by ionisation of the white silver to black silver oxide. Shortly after the discovery of X-rays the first breast cancer patient was treated by Emil Grubbe (Chicago, USA) using radiotherapy. Then, in 1886 (Paris, France), Henry Becquerel discovered radioactivity of natural sources by noticing emission of an unknown type of radiation coming from uranium salts. In 1898 Pierre and Marie Curie made several important discoveries: first, they isolated the radioactive elements polonium and radium, and many of their radioactive decay products. Radium was soon used for treatment of tumours. Then they discovered and characterized the ionizing properties of what they called 'radioactivity'. In 1920, Claude Régaud proved that fractionated therapy required a higher total dose to obtain tumour control, but caused fewer side effects. These discoveries mark the beginning of the use of radiation in medical science.

Ionising Radiation

Ionising radiation is a specific type of radiation that is energetic enough to cause ionisations upon interacting with atoms, resulting in detachment of electrons creating a charged ion. Types of ionising radiation are positively charged α-particles, negatively charged β-particles, neutrons, protons, and neutral γ-rays and Röntgen radiation (X-rays).

In this thesis we used both γ-radiation and Röntgen radiation (X-rays) for our radiobiological experiments. Both are forms of short-wavelength electromagnetic radiation emitting high-energetic photons and have a low linear-energy transfer (LET), producing low-density ionisations along sparse tracks. Photons produced by interactions between electrons and atoms are called γ-radiation, and photons from röntgen radiation originate from processes in the atomic nucleus. Although there is an overlap, in general γ-radiation possesses higher energy levels than röntgen radiation.

Radiation-induced DNA damage

The most important effects of ionising radiation on biological material (e.g. cell killing and mutation) are the consequence of ionisation, breakage and inadequate repair of the DNA. The DNA contains the code for many vital processes of the cell. Therefore, damage to the DNA may cause dysfunctioning and death of the cell.

Ionisations and excitations resulting from exposure to ionising radiation can cause damage to DNA due to direct interaction between the ionising radiation and the DNA molecules and, indirectly, by water radicals, in particular hydroxyl radicals ($OH^{\bullet}$) generated from interaction of ionising radiation with water molecules. The majority

of radiation damage is considered to be caused by indirect effects due to the presence of high amounts of water molecules in living cells.

Types of DNA damage that can occur after ionising radiation include base damages, DNA single and double-strand breaks, and DNA-DNA or DNA-protein crosslinks. The most rigorous DNA damage is a complete break of the DNA double helix. These DNA double-strand breaks are considered to be the most important cause of cell death by ionising radiation.

Unrepaired or inadequately repaired DNA damage may lead to sterile cells, interphase or mitotic cell death. However, seemingly normal surviving cells could harbour DNA damages like gene mutations or chromosomal aberrations that in due time may give rise to dysfunction of the cell and to cancer cells.

Repair of radiation-induced DNA damage

Base damages and DNA single-strand breaks are largely repaired by the action of enzymes in the base excision repair pathway (BER), which is normally highly efficient. DNA mismatch repair (MMR) is a molecular repair system for recognizing and repairing erroneous insertion, deletion and miss-incorporation of bases that can arise during DNA replication and recombination, as well as repairing some forms of DNA damage caused by irradiation or chemotherapy [83].

Two mechanisms to repair DNA double-strand breaks are homologous recombination (HR) and non-homologous end joining (NHEJ). HR requires a non-damaged homologous template for the repair process, resulting in error-free DNA repair. NHEJ is a relatively quick but error-prone repair process in which the ends of loose DNA strands are joined together, whereby pieces of DNA may be deleted.

Several DNA repair mechanisms are identified that alone, or in combination, are involved in repair of crosslinking of DNA with DNA or proteins, including nucleotide excision repair, double-strand break repair, MMR and the Fanconi anaemia pathway [44, 89, 97, 154].

Clonogenic assay

Cell death due to DNA damage can be measured *in vitro* by the clonogenic assay [45, 114]. This is a common assay to determine the intrinsic radiosensitivity of many types of cells. This *in vitro* assay establishes the reproductive fate of irradiated cells. The clonogenic assay can also be used to establish reproductive cell survival after treatment with cytotoxic drugs, or combinations of irradiation and cytotoxic drugs. The clonogenic assay is based on the ability of single clonogenic cells to form a colony. A colony contains 50 cells or more and represents about 5 or 6 cell divisions. Following treatment, damaged cells can occasionally still divide a few times or survive as a sterile cell. Only those cells that are still reproductive and able to form a colony are considered to ultimately survive. The clonogenic capacity of a specific cell line is presented as plating efficiency (PE), which is the number of colonies formed per number of seeded cells. PE = S(D) = number of colonies formed/number of seeded cells (Figure 1), where *S* is defined as the survival data for a certain radiation

dose *D*. The plating efficiency varies between different cell lines and often even the untreated cells do not possess a 100% clonogenic capacity. To calculate the surviving fraction (SF) the ratio of the PE of treated and control cells is taken: PE treated cells/PE control cells (Figure 1).

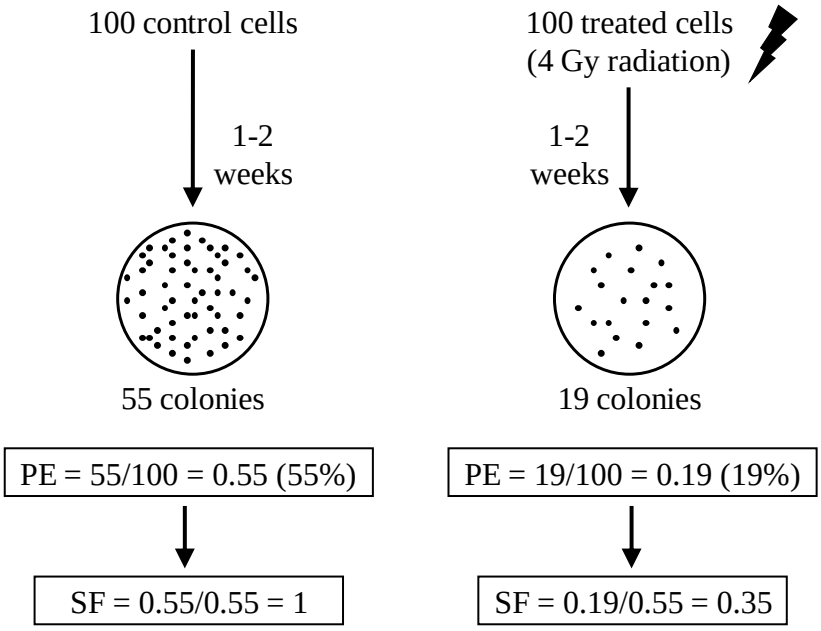

Figure 1. Fictitious data from a clonogenic assay for calculating plating efficiency (PE) and surviving fraction (SF) of control cells and cells treated with a radiation dose of 4 Gy.

Linear-quadratic model

A cell survival curve interpolates the surviving fractions (SF values) for an experiment wherein increasing radiation doses (Gy) were applied. In a survival curve, the SF is conventionally represented on a logarithmic scale on the y-axis. A well known and widely- used mathematical estimation of a cell survival curve is the linear-quadratic model (LQ model; [17]): $SF=S(D)/S(0)=\exp-(\alpha D+\beta D^2)$, presented in Figure 2. The LQ formula consists of two components: a linear component [$\exp(-\alpha D)$] and a quadratic component [$\exp(-\beta D^2)$]. The linear component describes the effects caused by single-hit events, proportional to the photon dose, while two-hit events are responsible for the quadratic component, proportional to the square of the dose. Lethal damages caused by single-track ionising particles are called single-hit events, whereas two-hit events are lethal lesions caused by interaction between sublethal damages from two different tracks. Examples of two survival curves for two different cell lines (D384 and VU-122) are given in Figure 3.

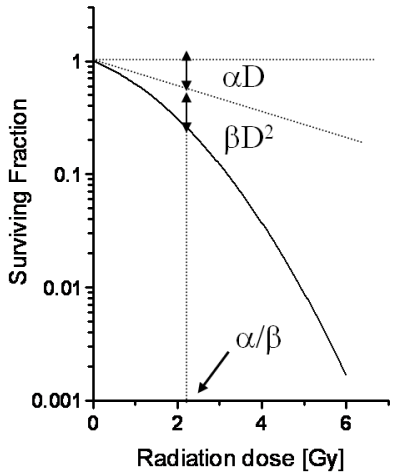

Figure 2. The linear-quadratic model (LQ model) for analyzing the relationship between cell survival and radiation dose.

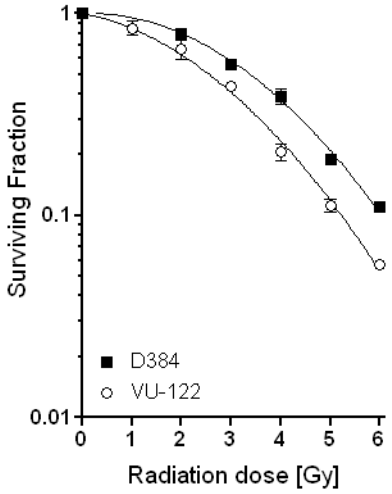

Figure 3. Typical examples of two survival curves for the cell lines D384 (■; n=6) and VU-122 (○; n=4).

The shape of the curve is determined by the α/β ratio, which is the dose of radiation at which the linear contribution to damage equals the quadratic contribution (Figure 2). For instance, the α/β ratio can be used as indicator for the sensitivity of cells to fractionated radiation treatment [1]. Fractionated radiation uses multiple fractions of low doses of radiation. Due to the time between the fractions, cells are allowed to repair damage and so reduce persistent DNA damage and cell death, as demonstrated in Figure 4.

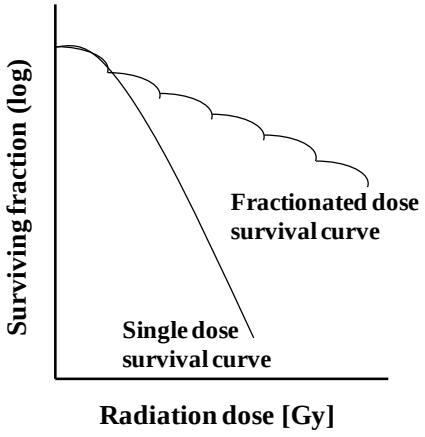

Figure 4. Survival curves showing the effects of fractionated radiation compared to single dose radiation on cell survival.

1.2 Glioma

Glioblastoma multiforme (GBM) is the most frequent and aggressive glioma (primary brain tumour) in adults. Despite extensive treatment, patients seldom survive a GBM for longer periods of time. Finding ways to improve therapy of GBM is warranted.

Glioma biology

Gliomas are the most frequent primary brain tumours that arise from the glial cells of the brain. Glial cells are the supportive cells in the central nervous system. The glial cells greatly outnumber the neurons in the brain, and the three types of glial cells are astrocytes, oligodendrocytes, and ependymal cells. Astrocytes perform many functions, including maintenance of the extracellular environment, recycling of released neurotransmitters, and regulation of blood flow due to changes in blood vessel diameter occurring with vasoconstriction and vasodilatation [77]. Oligodendrocytes are cells that provide myelination of the axons in the central nervous system. Ependymal cells cover up the cavities of the central nervous system (the ventricles) and create and secrete cerebrospinal fluid (CSF).

Astrocytomas, oligodendrogliomas and ependymomas, a very rare subtype of glioma, originate from astrocytes, oligodendrocytes and ependymal cells, respectively. Classification according to the 2000 World Health Organisation (WHO) scheme divides the glioma into four groups: astrocytomas (grades I-IV), oligodendrogliomas (grade II and III), mixed oligoastrocytomas (grade II and III), and ependymomas (grade I, II and III) [78]. Grades I and II glioma make up the low-grade gliomas. The high-grade or malignant gliomas consist of grades III and IV gliomas [78].

The most frequent and malignant glioma is the glioblastoma multiforme, a high-grade astrocytoma (grade IV) characterised by rapid, invasive growth, migratory behaviour, dedifferentiation, a heterogeneous tumour cell population, extensive vascularisation and necrosis. Despite their malignant behaviour, GBM (and glioma in general) seldom metastasise.

Median survival times for the low-grade astrocytoma grade II and the oligodendroglioma grade II are 5.6 and 11.6 years, respectively [105]. Median survival times of the higher-grade astrocytoma grade III, oligodendroglioma grade III, oligoastrocytoma III and GBM are 1.6, 3.5, and 2.8 years, and 14.6 months, respectively [105, 125, 138].

Important prognostic parameters for patients with an astrocytoma are age and histological grading of the tumour. A negative correlation exists between age and patient survival, and patients with higher grade astrocytomas have worse prognosis than patients with lower grade astrocytomas. Genetic changes in the tumour are another prognostic parameter as, for instance, demonstrated by studies from Leenstra et al. and Lin et al. [84, 85, 87]. These studies showed that the status of loss of heterozygosity (LOH) at chromosome 10 can be of importance for patient prognosis.

Glioblastoma multiforme genetics

The following chapter focuses on the genetic characteristics of GBM. Two major types of GBM are distinguished, each with a different course of disease and different genetic changes. GBM can either develop rapidly (primary (*de novo*) GBM) without any previous history of a lower-grade tumour, or gradually progress from lower-grade astrocytomas (secondary GBM). These two types of GBM differ regarding prognosis, patients' age and the genetic pathway through which these tumours developed [3, 33, 78]. Secondary GBM are more commonly found in younger patients, and primary GBM occur frequently in the elderly [3, 33, 78]. Because of their clinical history, patients with a secondary GBM have better prognosis than patients with a primary GBM.

Primary GBM prevail (>90%) and are genetically characterised by epidermal growth factor receptor (*EGFR*; #7p12) amplification and EGFR overexpression, phosphatase and tensin homolog (*PTEN*; #10q23) mutations, cyclin-dependent kinase inhibitor 2A (*p16/CDKN2A*; #9p21*)* deletion and LOH of chromosome arms 10p and 10q [3, 33, 79, 80, 124, 142, 144 149].

Secondary glioblastomas are characterised by mutations and LOH of the *TP53* gene (#17p13) and overexpression of platelet-derived growth factor A (PDGF-A; #7p22) and platelet-derived growth factor receptor α (PDGFR-α; #4q12) [3, 33, 79, 80, 124, 144 149]. These genetic changes are early events in the process towards a secondary glioblastoma. Other frequent genetic alterations that often (later) occur in the progression to a higher grade anaplastic astrocytoma are mutations in the retinoblastoma gene (*RB1*; #13q14), amplification of the cyclin-dependent kinase 4 gene (*CDK4*; #12q14), *p16/CDKN2A* deletion and LOH of chromosome arms 13q, 19q and 22q [3, 79, 124, 144]. The transition from an anaplastic astrocytoma into a secondary GBM is often accompanied by LOH of chromosome arm 10q [3, 79].

Glioblastoma multiforme therapy

High-grade gliomas are usually highly infiltrative tumours that are often difficult to remove completely by neurosurgery. Surgery normally consists of removal of as much of the tumour as possible. However, complete neurosurgical resection of the tumour is often not possible due to the invasive and migratory behaviour of the tumour, the risk of normal brain tissue injury, and due to problems with the exact extent of residual tumour during surgery. Frequently, the condition of the patient and/or the location of the tumour, only allows stereotactic tumour biopsy taken for histopathological diagnosis. Neurosurgery is pivotal for diagnosis and instant relief of symptoms, and the median survival after neurosurgery alone is 3.5 months (Table 1).

Radiotherapy is the single most effective treatment of patients with a malignant glioma. Radiation dose-escalation studies in the late 1970s have shown that increasing the total physical doses of radiotherapy improved the median survival from 3.5 months after best supportive care to 10-12 months with 60 Gy in 30 fractions of 2

Gy. Subsequent trials did not show a better tumour control for total doses above 60 Gy, but showed more complications.

Table 1. Historical improvement of survival in malignant glioma.

	RTOG/ECOG-trials (1978-1987)*				**EORTC 26981 (2005)****	
Treatment protocol	Surgery alone (S)	S + 50 Gy	S + 55 Gy	S + 60 Gy	S + 60 Gy	S + 60 Gy + TMZ
Median survival (months)	3.5	6.5	8.5	10	12	14

*[86, 145, 146]; **[138]

Fractionated radiation (30 x 2 Gy) is less efficient in cell killing than application of a single dose of 60 Gy would be. However, using fractionated radiation, a higher total dose can be applied due to the tissue-sparing effects of multiple time-spaced fractions (Figure 5). Because normal tissue cells are generally better capable of repairing radiation-induced DNA damage than tumour cells, a therapeutic window is obtained.

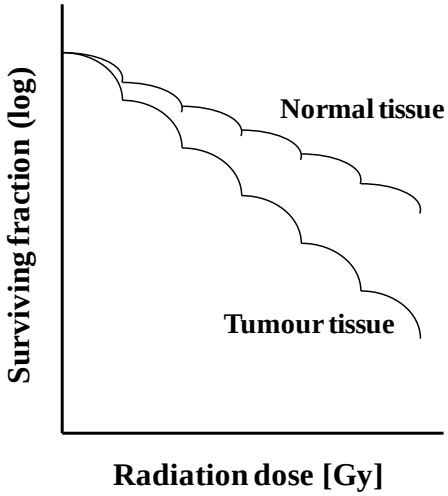

Figure 5. Survival curves showing the effects of fractionated radiation on normal tissue compared to tumour tissue.

Despite neurosurgery and high-dose radiotherapy, almost all glioma patients have a high risk of recurrence. Recurrences of high-grade astrocytomas occur in approximately 90% of the cases within a margin of 2-3 cm from the original tumour

site [2, 64, 147] and the lower-grade glioma will eventually recur as the same or higher-grade glioma [78].

Even with treatment consisting of surgery and postoperative radiotherapy, survival of patients with a GBM remained poor. After two decades of disappointing results, the study of Stupp et al. showed an improved survival after standard 60 Gy fractionated radiation therapy combined with concomitant and adjuvant temozolomide (TMZ) [138]. Fractionated irradiation was delivered in fractions of 2 Gy for 5 days/week during 6 weeks (total dose of 60 Gy). TMZ (75 mg/m^2) was given daily during the radiotherapy program, followed by six cycles of adjuvant TMZ (150-200 mg/m^2) for 5 days during each cycle of 28 days [138]. This randomised phase III trial demonstrated a significant increase in median survival from 12 to 14 months and an increase in the two-year survival rate [138]. Benefits of TMZ with radiotherapy lasted throughout 5-years of follow-up, with a survival rate of 9.8% versus 1.9% after radiotherapy alone [139]. Since the positive outcome of the trial, TMZ has become (at present) part of the standard therapy for patients with a newly diagnosed GBM.

Compared to the gain from radiotherapy in the late 1970s, the gain from combination therapy with TMZ is relatively small: nevertheless, more than 90% of these patients eventually die from this devastating tumour. However, the Stupp trial provided hope that the combination of radiotherapy with new drugs may further improve clinical results or the outcome of therapy. Such hopes motivated the studies described in this thesis, to better understand the biology of malignant gliomas and to improve the use of combined modality treatment.

1.3 The DNA alkylating drug temozolomide

The therapeutic potential of TMZ [138], concomitantly and adjuvant to radiotherapy, has made this combined modality treatment the standard of care for patients with a GBM. However, it remains unclear whether the therapeutic benefit of additional TMZ is caused by a synergistic or by a mere additive effect. Therefore, the radiosensitising potential of TMZ alone, or combined with other drugs, became an important focus of our research and was explored in the three studies presented in Chapters 4, 5 and 6. Furthermore, Chapter 3 investigates whether the response of tumour cell lines to TMZ can be predicted.

Temozolomide: mechanism of action

TMZ is a chemotherapeutic prodrug which, under physiological conditions, metabolises by spontaneous hydrolysis into its active metabolite 5-(3-methyl)-1-triazen-1-yl)imidazole-4-carboxamide (MTIC) [103]. MTIC is an unstable alkylating compound that will disintegrate within minutes [119]. Degradation of MTIC leads to the reactive compound, a methyldiazonium ion, which activity depends on transfer of the methyl group to DNA. TMZ is rapidly and almost completely absorbed after oral administration (with a plasma half-life of 74-110 min) and has a good tissue distribution, including the ability to cross the blood-brain barrier [98, 103]. TMZ has

a limited *in vitro* half-life of less than 30 min in plasma and around 75 min in phosphate buffer [137].

Methylation of the DNA by MTIC results in approximately 5-10% O^6-methylguanine adducts [103, 106, 123]. Although representing a small amount, the O^6-methylguanine adducts are considered to be responsible for the cytotoxic effect of TMZ [13, 141]. When not repaired, O^6-methylguanine adducts result in an incorporation of a thymidine instead of a cytosine opposite of the O^6-methylguanine during DNA replication. Futile attempts of the MMR system to find a complementary base for O^6-methylguanine will lead to ineffective replication and repair cycles [71, 98]. Mismatch repair proteins are required for the recognition and repair of faulty bases in the DNA. The faulty attempts of the MMR process to repair O^6-methylguanine adducts increases the amount of DNA double-strand breaks and eventually leads to cell death [88, 98, 104].

Anti-tumour effects of temozolomide

TMZ can induce a G_2/M arrest leading to senescence (p53 wild-type cells) or mitotic catastrophe (p53 deficient cells) [58]. On the other hand, TMZ can induce apoptosis via O^6-methylguanine lesions in human glioma cells [121], and in human glioma cells cultured as multicellular spheroids [52]. An important factor in deciding the fate of glioma cells with TMZ-induced O^6-methylguanine lesions is the status of the p53 protein [57-59, 121, 135]; another important factor is played by the MMR [8, 32, 60, 67, 88, 95]. A deficient MMR and expression of functional p53 protein are associated with a TMZ resistant phenotype.

Temozolomide and radiation

To date, a number of studies have investigated the radiosensitising potential of TMZ in glioma cell lines [11, 18, 19, 29, 57, 73, 143, 151]; however, these studies were not uniform in their conclusions. Enhancement of the radiation response was demonstrated in several cell lines in a few studies, while no interaction (merely an additive effect) was found by others. Based on these contradictory investigations it is evident that TMZ can act as a radiosensitiser, but not in every cell line and/or under every experimental condition.

The DNA repair protein O^6-methylguanine-DNA methyltransferase

Three DNA-repair activities can affect the cytotoxicity of TMZ, i.e. O^6-methylguanine-DNA methyltransferase (MGMT), MMR, and BER/poly(ADP-ribose) polymerase (PARP) [103].

MGMT is a cytoprotective DNA repair protein that can remove the methyl group from the O^6 position of guanine. This action disables the repair protein and requires protein resynthesis to restore the repair capacity [98, 109]. Therefore, the presence of this repair protein may undo (in part) the cytotoxic effect caused by alkylating agents,

resulting in resistance and shorter survival times of patients [4, 9, 10, 13, 43, 46, 55, 56, 61, 63, 71, 129].

Human gliomas demonstrate a heterogeneous expression of the MGMT protein both interindividual and intercellular [5, 27, 128]. In addition, transcriptional silencing of the *MGMT* gene by hypermethylation of the CpG dinucleotides (CPGs) in the promoter region has been demonstrated in a variety of tumour cell lines as well as in human tumour tissues, including glioma [30, 42, 116, 150]. However, caution is warranted since methylation of the *MGMT* promoter can vary within the same tumour after treatment [107]. Furthermore, two other studies have shown that the *MGMT* gene can be induced by methylating agents, dexamethason and radiation [20, 49]. The cloned human *MGMT* promoter in a rat hepatoma cell line was found to be induced by two methylating agents, ionising radiation and dexamethason [49], and irradiation-induced expression of MGMT has been observed in the liver of rats [20].

O^6-methylguanine-DNA methyltransferase as prognostic factor

Several patient studies have investigated the relationship between the role of MGMT and the clinical response of patients treated with radiotherapy with or without alkylating agents like TMZ. Methylation of the CpGs in the promoter region of the *MGMT* gene as well as absence of the MGMT protein have been associated with a good clinical response to alkylating agents in general and, in particular, to TZM in patients with an anaplastic astrocytoma or GBM [25, 37, 39, 43, 55, 56, 99, 108, 122, 134].

The MGMT status is an important prognostic factor for survival in GBM patients, both in patients that only received radiotherapy, and more so in patients that received radiotherapy combined with TMZ. However, uncertainty still exists concerning the best way of determining the MGMT status, especially since the few studies that compared different approaches demonstrated a lack of correlation between expression of the MGMT protein and methylation of the *MGMT* promoter [12, 65, 113, 120, 156]. Nevertheless, a recent study showed a significant correlation between *MGMT* promoter methylation status and MGMT protein expression levels [134], but this association was not followed by a small subgroup of the samples.

1.4 The histone deacetylase inhibitor valproic acid

The anti-epileptic drug, valproic acid (VPA) is frequently prescribed in glioma patients with epileptic seizures. VPA is an inhibitor of histone deacetylase, and thereby a potential anti-cancer drug and radiosensitiser. During the treatment of glioma patients, VPA might interact with other drugs (e.g. TMZ) or radiation therapy from the given treatment protocol. Therefore, the interaction of TMZ, VPA and irradiation was investigated in two glioma cell lines; the results are presented in Chapter 5.

Valproic acid as anti-epileptic drug

VPA (2-n-propylpentanoic acid), a small-branched fatty acid, is a worldwide commonly prescribed broad-spectrum anti-epileptic drug (AED) used for the treatment of generalised and partial seizures.

VPA was synthesised in 1882 by Burton as an organic solvent [14]. However, it was not until 1963 that the anti-epileptic potential of VPA was fortuitously discovered by Eymard, when VPA was used as an organic solvent for compounds that were tested for their anti-epileptic activity. Unexpectedly, VPA itself turned out to be an effective inhibitor of drug-induced seizures, as reported by Meunier et al. in 1963 [93].

VPA has good oral bioavailability and is in general well tolerated. The most serious side effects are teratogenicity and liver failure [38, 81, 90].

VPA is a broad-spectrum anti-epileptic drug acting its pharmacological effects through multiple mechanisms. VPA is known to affect the function of the neurotransmitter γ-aminobutyric acid (GABA), to attenuate neuronal excitation mediated by the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, to reduce the release of γ-hydroxybutyrate (GHB), and to block voltage-gated sodium channels and T-type calcium channels [81, 90].

Histone deacetylases and histone deacetylase inhibitors

Chromatin is the combination of DNA, histones, and other proteins that make up chromosomes. An important function of histones is to modify the structure of chromatin, e.g. to condense DNA into a smaller volume to fit in the cell, or to extend the chromatin to allow DNA replication and cell division, and to control gene expression [35]. The shape of chromatin is chemically modified by methylation and acetylation of the histones. The balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities determines the net level of acetylation. Acetylation of amino acid residues in the tails of the histones by HATs is associated with a relaxed chromatin structure (euchromatin) allowing DNA to be accessible for transcriptional activity. HDACs can remove the acetyl groups from the histone tails. Deacetylation results in a compacted or closed chromatin structure (heterochromatin) that is associated with repression of transcription.

HDAC activity has long been associated with cancer development [47]. The ability of HDACs to modulate chromatin structure enables HDACs to regulate expression of numerous genes that are involved in cancer initiation and progression. Furthermore, HDACs also deacetylate other proteins of which many are involved in cancer development.

Due to the increasing knowledge about their role in cancer development, HDACs have been considered as potential targets for cancer treatment. Inhibition of HDACs can result in induction of growth arrest, cell differentiation and apoptosis of cancer cells *in vitro* and *in vivo* [35, 69, 91]. Furthermore, HDAC inhibitors are reported to

enhance the effect of several anticancer drugs targeting the DNA in different human cancer cell lines [74].

Valproic acid as histone deacetylase inhibitor

VPA is an effective HDAC inhibitor at therapeutic levels [48, 53, 112]. Linked to the HDAC inhibiting activity are the effects of VPA to inhibit cell proliferation and to induce cell differentiation in various human tumour cell types [48, 81]. VPA has also been reported to induce apoptosis in and to increase immunogenicity of cultured cancer cells [81]. Similar to other HDAC inhibitors, VPA has been shown in human glioma cell lines to increase the sensitivity of several anticancer drugs that target the DNA [28, 31]. Furthermore, VPA also has the potential to demethylate DNA [36, 94]. If VPA does cause demethylation of the promoter of the *MGMT* gene, the expression of the MGMT protein might be affected, causing resistance to TMZ. Since both VPA and TMZ are common drugs given to patients with a GBM, VPA could then antagonise the effect of TMZ, contraindicating prescription of TMZ to these patients.

Valproic acid and radiation

Several studies on various tumour types have demonstrated the ability of different HDAC inhibitors to enhance the radiation response both *in vitro* and *in vivo* [15, 23, 40, 41, 68, 75, 76]. Also, VPA has shown to act as radiosensitiser in a variety of tumour cell types [7, 22, 34, 69], although so far only two *in vitro* studies have been conducted on glioma cell lines [16, 24].

1.5 The cyclooxygenase-2 inhibitor meloxicam

Cyclooxygenase-2 (COX-2) proteins are potential targets for glioma therapy (Figure 6). *In vitro* and *in vivo* studies have shown many anti-tumour effects by COX-2 inhibitors in a number of tumour types. The anti-tumour potential may be enhanced by combination with chemotherapeutic drugs and/or radiation therapy. The interactions between the COX-2 inhibitor meloxicam (MLC), TMZ and radiation were investigated and are presented in Chapter 6.

Cyclooxygenases

Cyclooxygenases are key enzymes that are responsible for the rate-limiting step of the conversion of arachidonic acid into prostaglandin H_2 (PGH_2) during synthesis of the prostanoids. The cyclooxygenase-1 (COX-1) protein is constitutively expressed in normal tissues and involved in normal physiological processes (house-keeping enzyme), while the inducible COX-2 protein is involved in pathological conditions and cancer. COX-2 has shown to stimulate proliferation, migration and invasion, neovascularisation, and inhibition of apoptosis and can be induced, for instance, by cytokines, growth factors, and radiation [130, 136]. A third isoenzym,

cyclooxygenase-3 (COX-3), a splice variant of COX-1, has also been identified [21]. Expression of COX-3 mRNA was mainly found in the cerebral cortex and the heart; however, the role of COX-3 is currently still unknown [21, 131].

Cyclooxygenase-2

Generally, the inducible COX-2 enzyme is not (or hardly) present in normal tissues [130, 131]. Meanwhile, overexpression of the COX-2 protein has been detected in a variety of tumour types, including breast, colorectal, lung, pancreatic and prostate cancer, and gliomas [26, 131, 157]. COX-2 overexpresssion has been associated with malignancy and poor patient prognosis [54, 66, 127, 131]. Also in gliomas a correlation was found between the expression of the COX-2 protein with a higher malignancy of tumours and shorter survival times [54, 66, 110, 127]. Compared to lower grade gliomas, GBM often contain the highest level of COX-2 protein. The COX-2 protein is therefore a potential therapeutic target for treatment of glioma, especially GBM.

Cyclooxygenase-2 inhibitors

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory drugs, and to treat pain and fever. Most NSAIDs are COX inhibitors of which the first generation that was developed were non-selective and targeted both COX-1 and COX-2. Examples of these non-selective COX inhibitors are aspirin, ibuprofen, naproxen and sulindac. A new generation of NSAIDs has been developed: the COX-2 selective inhibitors. Exclusive inhibition of COX-2 would retain the positive effects of the NSAIDs, but with reduction of the gastrointestinal adverse events caused by COX-1 mediated processes. Examples of the selective COX-2 inhibitors are celecoxib, rofecoxib, SC-236 and NS-398. However, concerns were raised about the risks of myocardial infarction after long-term/chronic intake of these drugs [131].

COX-2 inhibitors have demonstrated anti-tumour properties in a variety of tumour types both *in vitro* and *in vivo* [66, 92, 131]. The mechanisms involved in their anti-tumour effects include induction of apoptosis, inhibition of cell proliferation, and inhibition of angiogenesis. Several studies have reported that COX-2 inhibitors can enhance the effects caused by different chemotherapeutic drugs [96, 102, 133].

Interestingly, several studies have reported that the specific COX-2 inhibitors induced their anti-tumour actions without the involvement of COX-2 activity or prostaglandin synthesis, but by means of COX-2 independent mechanisms [50, 51, 70, 101, 131, 132, 140, 148]. These COX-2 independent mechanisms by the specific COX-2 inhibitors were found in a number of cancer cell lines of different origin and in mouse tumour models.

Meloxicam as cyclooxygenase-2 inhibitor

MLC was developed in 1977 by Boehringer Ingelheim as a new NSAID. Although often mentioned as a selective COX-2 inhibitor, it preferentially inhibits COX-2 over COX-1. A certain concentration responsible for 80% inhibition (IC_{80}) of COX-2 is approximately equal to 20% inhibition (IC_{20}) of COX-1.

Like other COX-2 inhibitors, MLC has demonstrated a number of anti-tumour properties in cell lines and animal models of different tumour types [62, 72, 101, 153, 155]. The anti-tumour actions that MLC demonstrated in these studies were inhibition of tumour growth, invasiveness, metastasis, angiogenesis and induction of apoptosis.

Meloxicam and radiation

Several COX-2 inhibitors have been shown to enhance the radiation response in cell lines and tumours of many cancer types [100, 111, 115, 117, 118, 126, 152]. To date only a few studies have explored the role of MLC in human glioma [6, 82]. These studies, all from our own group, investigated the radiosensitisation potential of the COX-2 inhibitors MLC, NS-398 and celecoxib. These studies showed that MLC can affect the radiation response in several glioma cell cultures.

1.6 Aims and outline of the thesis

Treatment of patients with a GBM, consisting of surgery, radiotherapy and chemotherapy is beneficial compared to no treatment at all. Nevertheless, despite this aggressive therapy, improvement of survival by the current therapy protocol is limited, and median patient survival remains at merely 14 months, with 5-year overall survival rates of around 10%. This poor prognosis challenges us to search for new therapies or treatment strategies to improve GBM therapy and patient survival rates.

Therefore, the main objective of the studies described in this thesis was to search (on a fundamental level) for ways to improve glioma therapy. As an approach, several targets inside the glioma tumour cell were selected to test their potential as therapeutic target with drugs influencing their functioning; in particular, the response of the tumour cells after combining these drugs with radiotherapy (Figure 6).

Most of the investigations in this thesis used cell lines (both primary and established) of high-grade glioma, mainly GBM. In two of the studies, genetic profiles were determined from tumour samples and primary cell lines. In the search to improve GBM therapy, combinations of novel (MLC) and clinically approved drugs (TMZ/VPA) were tested in combination with single or fractionated doses of γ-radiation or X-rays.

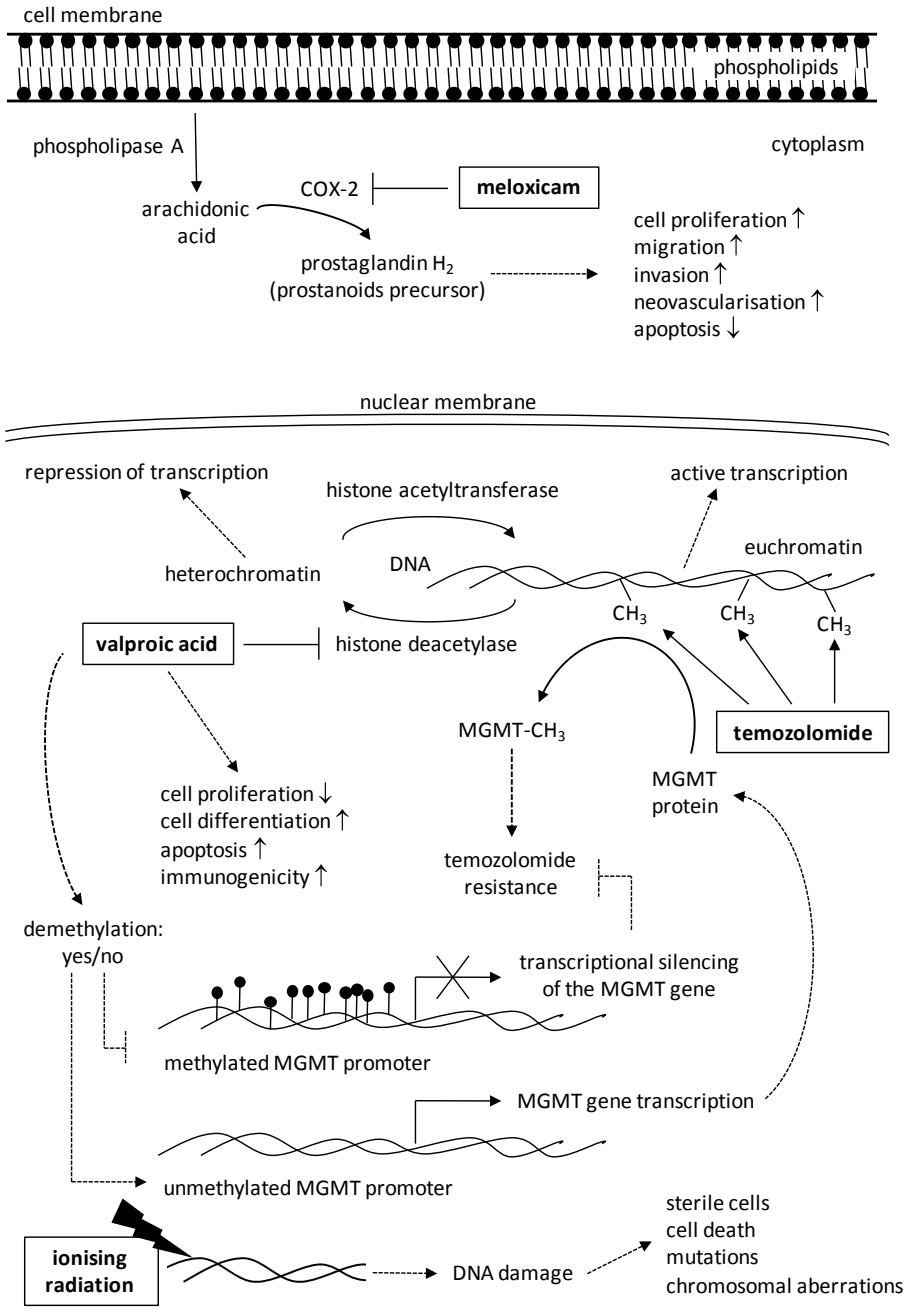

Figure 6. Representation of the cancer cell with several molecular targets that were selected for therapeutic intervention with γ-radiation and novel (meloxicam) and clinically approved (temozolomide and valproic acid) drugs showing actions, interactions and resulting consequences.

Despite treatment, recurrences of GBM occur in the majority of patients and often develop near the original tumour site within a short period of time. Chapter 2 focuses on two patients who developed a second GBM after a relatively long time interval. The second GBM was located outside the previously irradiated target area at a remote distance from the primary tumour location. Genetic profiles of the tumours were compared to discriminate between distant recurrence and true second primary GBM. Knowledge about the origin of such tumours may have implications for future treatment.

The DNA repair enzyme MGMT has proven to be a prognostic factor for predicting the clinical response of patients with an anaplastic astrocytoma or GBM that received TMZ in their treatment protocol. However, questions remain about the best way to determine the MGMT status, via determination of gene promoter methylation or protein expression levels. Therefore, the purpose of the study presented in Chapter 3 was to determine whether the cytotoxic response to TMZ in a series of tumour cell lines, mostly derived from human GBM, is associated with either the expression of the MGMT protein and/or the methylation of the *MGMT* promoter.

Despite the role of TMZ in the standard clinical treatment protocol for GBM, limited information is available about the nature of the interaction leading to therapeutic benefit Therefore, the radiosensitising potential of TMZ was investigated in the study presented in Chapter 4 for human GBM cell lines using single-dose and fractionated γ-irradiation. The three cell lines used were long-term primary cell lines created in our laboratory; the cell lines were sensitive to TMZ.

In glioma patients, the commonly prescribed anti-epileptic drug VPA has the potential to demethylate DNA. Demethylation of the promoter of the *MGMT* gene might influence the expression of the MGMT protein due to losing methylation of the *MGMT* gene promoter, causing resistance to TMZ. VPA could then antagonise the effect of TMZ, contraindicating prescription of TMZ to GBM patients. Therefore, the interactions between VPA, TMZ and γ-radiation were investigated in the study presented in Chapter 5 for two established human glioma cell lines that differ in TMZ sensitivity caused by the absence or presence of the MGMT protein.

The COX-2 protein, often highly expressed in GBM, may be a potential therapeutic target for the treatment of these tumours. Earlier investigations in our laboratory led to the discovery of a promising COX-2 inhibitor, MLC, that can be a potent radiosensitiser in cultures of human glioma cells. Combining MLC with the present therapeutic modalities radiotherapy and TMZ is therefore warranted. Chapter 6 presents the investigations of the trimodal combination in three established human glioma cell lines that were sensitive to TMZ due to absence of the MGMT protein.

Finally, some topics relevant to the studies presented in this thesis are discussed and placed in perspective within a general discussion (Chapter 7), followed by a summary in English and Dutch (Chapter 8).

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